Respiratory Medicine (2009) **103**, 589–594

available at www.sciencedirect.com

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Gas6 evaluation in patients with acute dyspnea due to suspected pulmonary embolism

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Received 30 July 2008; accepted 21 October 2008
Available online 25 November 2008

KEYWORDS
Acute dyspnea;
Gas6;
Pulmonary infections;
Pulmonary embolism

Summary
Background: Gas6 protein is involved in pulmonary embolism (PE) and acute inflammation in animal models.
Methods: We enrolled 82 consecutive patients with acute dyspnea and suspected PE (Geneva score with high (HCP) or low/intermediate clinical probability (LICP) + D-dimer $\geq$0.5 μg/mL) and 29 age-matched healthy volunteers. According to clinical and instrumental evaluations the following diagnoses were obtained: heart failure (HF), pulmonary or systemic infection (I), PE, or no illness (N). Twenty-two patients were excluded due to oral anticoagulation (9), lack of CT angiography or pulmonary scintigraphy (6), plasma creatinine $\geq$3 mg/dL (3), and pulmonary cancer (4). Plasma Gas6 was measured with a validated enzyme-linked immunoassay. Non-parametric tests and accuracy measures were calculated.
Results: Out of 60 patients included, 8 were N, 12 HF, 11 I and 29 PE. Gas6 median value in the N group (20.4 ng/mL, interquartile range 17.6–21.6) matched that of healthy volunteers, 19.1 (17.2–21.4). Median Gas6 values in HF, 26.4 (21.6–33.3) and I groups, 34.1 (30.0–38.7), were significantly higher than those in PE 18.2 (16.3–23.3) or N (Kruskal–Wallis test $p \leq 0.05$) groups. Gas6 test improved PE diagnosis with an area under the curve of 0.80 and 0.91 (in all and LICP patients). A 24 ng/mL threshold excluded PE in 33% of LICP patients without loosing any diagnosis.

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0954-6111/$ - see front matter
doi:10.1016/j.rmed.2008.10.018

Conclusions: The data link Gas6 protein to infection/inflammation, but not to PE, in humans. Gas6 assay was useful in PE diagnosis, improving D-dimer accuracy particularly in LICP patients, and limiting false positives.

Introduction

The differential diagnosis of acute dyspnea is often challenging because it is difficult to identify the underlying disease rapidly. Only a few biomarkers are reliable;[1] even more so in relation to pulmonary embolism (PE), in which the only reliable plasma marker is D-dimer.[2] Gas6 (growth-arrest-specific protein 6) is a 75-kDa protein with 44% homology to coagulation protein S.[3,4] Both proteins bind to tyrosine kinase Tyro3 family receptors (Axl, Tyro-3, Mer) but Gas6 has greater affinity for Axl.[5–8] These receptors have been found to be coexpressed with Gas6 protein in CNS, endothelium, platelets and monocytes.[9–12]

There is evidence to support Gas6 protein involvement in the pathogenesis of PE: gas6 −/− mice are protected from induced venous thromboembolism because they develop smaller and fewer organized intravascular thrombi comparing to wild-type animals. Mice treated with anti-Gas6 antibodies display the same phenotype, and are equally protected against fatal thromboembolism.[12,13] Other reports indicate a possible role for Gas6 protein in control of innate immunity and in inflammation processes. Axl −/−, Mer −/− and Tyro 3 −/− mice display hyper-activation of monocytes/macrophages with a higher susceptibility to endotoxic shock and consequent mortality; their monocytes react with an excessive secretion of tumor necrosis factor (TNF)-α and interleukin (IL)-6 after lipo-polysaccharides (LPS) challenge.[14,15] Some authors have also suggested the potential involvement of Gas6 in acute inflammation in humans because plasma Gas6 concentrations were found to be significantly increased in patients affected by septic shock in intensive care units.[16,17] Data relating to the plasma concentration of Gas6 in humans with PE are not available.

PE and pulmonary or systemic infections are common causes of acute dyspnea. We tried to evaluate if plasma Gas6 concentration varied among different causes of acute dyspnea; this could aid differential diagnosis in patients with acute dyspnea and suspected PE.

Methods

Patients

From March 2005 to August 2008 we enrolled 82 consecutive patients with acute dyspnea due to suspected PE in the present observational study. Inclusion criteria were: acute dyspnea onset within the last 24 h and one of two findings: (1) Geneva[18] score $\geq$9 (high clinical probability for PE—HCP); or (2) Geneva score <9 (low/intermediate clinical probability for PE—LICP) together with plasma D-dimer value $\geq$0.5 μg/mL. Patients were aged >18 years and gave written informed consent. At the beginning of the enrollment a review board was not required for observational studies. Patients were excluded if plasma creatinine was $\geq$3 mg/dL, if they suffered from a primitive or secondary pulmonary cancer, or if they were being treated with oral warfarin.

All patients included were evaluated for PE diagnosis according to in-use medical practice with either thorax contrast-enhanced multidetector spiral computed tomography (CT angiography) or with pulmonary perfusion scintigraphy (111 MBq of ^{99m}Tc-MAA mean dose).

At enrollment, before any imaging exam, a 20-mL blood sample was drawn from enrolled patients. Plasma and serum samples were obtained within 1 h after centrifugation and stored at −80 °C. Gas6 was measured at the end of the enrollment.

Table 1 Criteria for the dyspnea-related diagnoses.

Diagnosis	Definition	Criteria
N	Absence on acute cardiac or pulmonary disease	Absence of clinical or instrumental evidence of acute cardiac or pulmonary disease (all patients discharged within 12 h from the emergency department of our hospital)
HF	Worsening chronic or *de novo* heart failure or pulmonary edema with elevated, normal or reduced systolic blood pressure[28]	Clinical signs of systemic or pulmonary congestion + chest radiograph supportive of pulmonary congestion; or echocardiogram supportive of a reduced cardiac output[28]
I	Pulmonary infection, systemic infection, sepsis	One of the following: (a) clinical signs of infection (fever, chills) + blood or sputum cultures positive or chest radiograph supportive of pneumonia; (b) diagnosis of sepsis (not severe) according to ISDC criteria[29]
PE	Pulmonary embolism	Positive CT angiography of thorax or scintigraphy lung perfusion with high probability for PE confirmed by significant improvement or resolution of PE after at least 7 days of anticoagulation with low molecular weight heparin or oral warfarin

Clinical data and the other instrumental examination results were used to classify patients into one of four groups: absence of acute cardiopulmonary illness (for example anxiety) (N), acute heart failure (HF), pulmonary or systemic infection including chronic obstructive pulmonary disease (COPD) exacerbations (I), and pulmonary embolism (PE). The definitions and diagnostic criteria used for these groups are described in Table 1. Patients with a combination of causes of dyspnea (e.g. COPD exacerbation and acute heart failure) were not considered. Two physicians defined all the diagnoses independently. Discordant diagnoses were revaluated and assigned by consensus.

A group of 29 volunteers age-matched to patients were enrolled if they were healthy, except for arterial hypertension under treatment. Healthy controls had 20 mL of blood taken, and samples processed as described above.

Laboratory analysis

Blood gas data, without oxygen supplementation, and plasma creatinine were assayed in automated laboratory equipment (ADVIA 1650, Bayer Diagnostics Italia). D-dimer was assayed with VIDAS D-dimer enzyme-linked fluorescent assay on an automated immunoassay system (bioMérieux Italia).

Gas6 protein was measured with a sandwich enzyme-linked immunoassay (ELISA). The method has been validated according to Food and Drug Administration (FDA) guidelines[19] in a previous study (inter-assay and intra-assay %CVs were within 15%, mean recovery on 15 patients of 96%, lower limit of quantification—LLOQ—0.25 ng/mL).[20] Briefly, a 96-well plate (NUNC ImmunoPlates, UK) was coated overnight with anti-Gas6 primary antibody (Goat polyclonal affinity purified IgG, R&D Systems, Minneapolis, USA). Antigen was detected by a secondary biotin-conjugated antibody (Biotinylated anti-human Gas6 antibody, R&D Systems, USA) and a streptavidin–peroxidase conjugate (Sigma, USA) and TMB (3,3′,5,5′-tetramethylbenzidine, Sigma, USA). The reaction was blocked with 1.8 M sulfuric acid; absorbance was detected at 450 nm with a reference wavelength set at 570 nm. Optical density was fitted versus nominal concentration, applying a four-parameter logistic regression to the calibration curve prepared in Gas6-depleted human plasma with human recombinant Gas6 addition (R&D, USA).[20]

Statistical analysis

Data were analyzed with appropriate statistical software (Statsoft). The Shapiro–Wilk test was done to assess normality of each variable in any group. Median values, together with interquartile range (IQR), were reported. Median values of each group were compared with Kruskal–Wallis *H*-test (variance) and with Kruskal–Wallis test in case of multiple comparisons or with Mann–Whitney *U*-test for comparisons between two groups. For Gas6 and D-dimer accuracy, measurements were calculated at confidence intervals (CIs) of 95%, together with positive likelihood ratio.

Results

Out of the 82 patients enrolled, 60 were included; 8 were N, 12 had HF, 11 had I, and 29 had PE. Of the 22 excluded, 9 patients were already being treated with anticoagulation (p.o.), 6 patients lacked a CT angiography or evidence of PE resolution with pulmonary scintigraphy, 3 patients had plasma creatinine >3 mg/dL, and 4 patients suffered from a primitive pulmonary cancer. All patients included displayed D-dimer values higher than 0.5 μg/mL. All variables analyzed did not have a normal distribution in any diagnostic group (Shapiro–Wilk, $p > 0.05$).

Gas6 median concentration was equivalent in the N and healthy volunteers groups (20.4 ng/mL (17.6–21.6) and 19.1 (17.2–21.4) respectively) (Mann–Whitney *U*-test, $p =$ not significant (n.s.)). Gender did not influence Gas6 concentration within each group in healthy controls (Mann–Whitney *U*-test, $p =$ n.s.).

Table 2 details patients' characteristics; there were no significant differences in age and plasma creatinine concentration among groups (Kruskal–Wallis variance, $p > 0.1$). Table 2 and Figure 1 also report median plasma Gas6 concentration of each group. In non-parametric post-hoc comparisons, median plasma Gas6 concentration in HF group or I group patients was higher than in those of PE or N groups (Kruskal–Wallis test for multiple comparisons, N vs. HF $p = 0.05$, N vs. I $p < 0.002$, I vs. PE $p < 0.0001$, I vs. N $p < 0.002$). Plasma Gas6 concentration was also higher in the I group than the HF group though it was not significant, and was nearly identical compared to PE and N groups (Mann–Whitney *U*-test, $p = 0.9$).

We evaluated the usefulness of Gas6 as a diagnostic test for PE. D-dimer 0.5 μg/mL determined a 52% [95% CI 39–65] false positive rate. Gas6 testing on D-dimer positive subjects displayed high true positive and low false positive rates for several cut-offs tested with a greater performance in patients with LICP for PE (Geneva score <9) (Table 3). Moreover, as shown in receiver operator characteristics curve analysis of Figure 2, area under the curve (AUC) of Gas6 in PE diagnosis was 0.80 for all patients and 0.91 for

Table 2 Patient characteristics of dyspnea groups.

	N[8]	HF[12]	I[11]	PE[29]
Gas6 (ng/mL)	20.4 [17.6–21.6]	26.4 [21.6–33.3]	34.1 [30.0–38.7]	18.2 [16.3–23.3]
D-dimer (μg/mL)	0.80 [0.64–1.22]	1.16 [0.87–1.39]	1.41 [0.95–1.86]	1.89 [1.22–9.21]
PO_2 (mmHg)	75.6 [75.0–77.9]	69.3 [65.0–76.9]	61.4 [58.3–65.0]	61.5 [54.7–68.0]
PCO_2 (mmHg)	33.1 [31.6–34.6]	33.7 [30.1–36.0]	30.7 [26.6–37.3]	32.1 [30.0–33.9]
Creatinine (mg/dL)	0.9 [0.8–1.2]	1.3 [1.1–1.6]	1.4 [1.2–1.7]	1.1 [0.9–1.3]
Age (years)	66.5 [45.5–74]	74 [69.5–79]	80 [70–84]	75 [66.5–80]

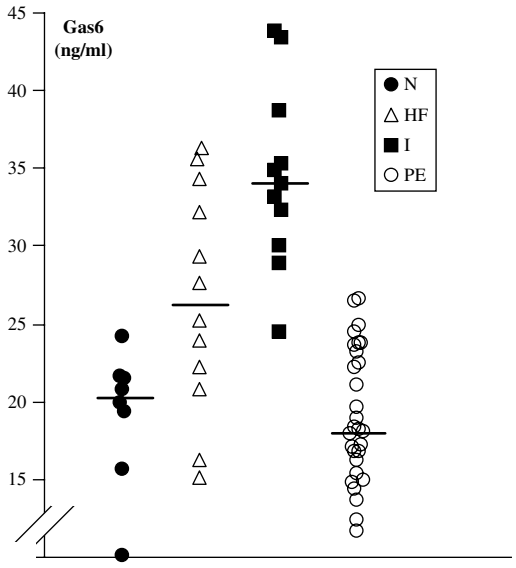

Figure 1 Plasma Gas6 concentration with median values reported as horizontal lines for any group analyzed. See text for statistical comparisons and explanations.

subjects with LICP for PE. Finally, given a Gas6 plasma level $\geq$24 ng/mL, PE could be excluded in 16 out of 48 (33%) patients with LICP for PE and D-dimer $\geq$0.5 μg/mL (Table 4).

Discussion

Since gas6 is involved in PE or systemic inflammation in animals,[12–15] we evaluated plasma Gas6 protein concentration in humans in relation to different causes of acute dyspnea.

We measured a median concentration of 19.1 ng/mL in healthy volunteers, which was nearly identical to that observed in young healthy donors in a previous study.[20] A group from Sweden obtained very similar results applying a different ELISA method.[21] Researchers from France obtained a Gas6 concentration of 52.0 ng/mL and 63.8 ng/mL in healthy men and women, respectively.[22] Although we cannot exclude ethnic or geographical variations, we suggest that differences in assay methods could explain this discrepancy.

Our results demonstrated that a Gas6 protein test could be useful for the differential diagnosis of acute dyspnea: Gas6 assay substantially increased D-dimer predictive value in defining PE diagnosis. This effect was even more apparent in patients with low to intermediate clinical probability for PE. This fact is important since the patients with low–intermediate probability for PE display a higher proportion of false positivity on the D-dimer test. Therefore these two tests used in combination could more accurately identify PE, helping the clinician to avoid further expensive instrumental work-up (pulmonary scintigraphy or CT angiography).

Plasma Gas6 concentration was increased by about two times the normal value in patients with acute dyspnea due to pulmonary or systemic infection. This finding confirms the hypothesis that Gas6 protein may be involved in acute inflammation: a monocyte–macrophage hyperactivation has been described in mice lacking Gas6 receptors[14,15] and plasma Gas6 concentration increases in patients with severe sepsis, proportionally to disease severity.[16,17] Thus Gas6 seems to be involved in macrophage function, being a possible biomarker of this activity.

We also demonstrated a 1.5-fold increase in plasma Gas6 concentration in subjects suffering from acute HF with respect to baseline values. The elevation of inflammation biomarkers and in particular of cytokines involved in innate immunity, such as TNF-α, in HF is well documented.[23–25] Gas6 elevation may be interpreted as an indicator of innate immunity activation in this setting.

Gas6 protein was found to be expressed in mouse platelets, secreted after platelet activation and bound to Gas6 receptors on the platelet surface, suggesting an autocrine stimulatory mechanism; moreover gas6 −/− mice were protected from fatal thromboembolism similar to wild-type mice treated with anti-Gas6 antibodies, without alteration in bleeding.[12,13] Platelet aggregates of Gas6 −/− mice are loosely packed with few contact sites, and are incompletely degranulated.[26] All these data suggest that Gas6 interaction with its receptors is important in thrombus stabilization, possibly through an outside-in signaling involving the integrin $\alpha_{IIb}\beta_3$; however the interaction of Gas6 with its receptors is involved only in amplifying the response of other platelet agonists but not determining platelet activation by itself, as is true for other molecules such as Eph kinases and ephrins or CD40 ligand.[26] Additionally, Gas6 binds to its receptors through a domain that needs a vitamin K dependent γ-carboxylation to be active, tightly linking this protein to hemostatic processes.[27] In contrast to the evidences exposed above,

Table 3 Accuracy of different Gas6 cut-offs in PE diagnosis.

All patients $n = 60$	Sensitivity (%)	62 [42–79]	86 [67–95]	100 [85–100]	100 [85–100]
	1 − specificity (%)	26 [13–45]	39 [22–58]	48 [31–67]	58 [39–75]
	Positive LR	2.4 [1.2–4.7]	2.2 [1.4–3.5]	2.1 [1.4–3.0]	1.7 [1.3–2.3]
	Negative LR	0.5 [0.3–0.8]	0.2 [0.1–0.6]	0 [0.0–0.0]	0 [0.0–0.0]
	Gas6 cut-off (ng/mL)	<21	<24	<27	<30
Patients with LICP $n = 48$	Sensitivity (%)	80 [56–93]	100 [80–100]	100 [80–100]	100 [80–100]
	1 − specificity (%)	29 [14–49]	43 [25–63]	50 [31–69]	61 [41–78]
	Positive LR	2.8 [1.5–5.2]	2.3 [1.5–3.6]	2.0 [1.4–3.0]	1.6 [1.2–2.1]
	Negative LR	0.2 [0.1–0.5]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]

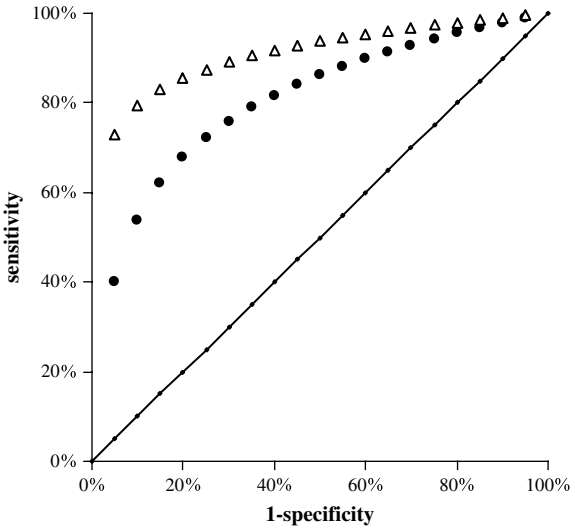

Figure 2 Receiver operator characteristics (ROC) curves of Gas6 testing in all patients included (black circles) and in subjects with low-intermediate probability for PE (open triangles). The equations of fitted ROC curves are: $y = 0.2\text{Ln}(x) + 1$, $R^2 = 0.99$, AUC = 0.80 (all patients) and $y = 0.09\text{Ln}(x) + 1$, $R^2 = 0.93$, AUC = 0.91 (low-intermediate probability for PE) respectively.

we found that PE does not influence plasma Gas6 concentration in humans. The simplest explanation for this discrepancy is that Gas6 may be important for thrombus formation but it is not overexpressed or released during platelet aggregation or during the coagulation process, perhaps acting as a membrane protein for platelets or endothelial cells.[12,13] It should also be considered that other authors have raised concerns about Gas6 function in human hemostasis: they failed to find Gas6 protein expression in human platelets, suggesting that it may not be as relevant in humans as it is in mice.[21] Our data seem to support the latter hypothesis; however, further research is needed to clarify Gas6 function in human hemostasis.

In conclusion, plasma Gas6 concentration is increased in patients with acute dyspnea due to HF and even more in patients with systemic or pulmonary infection; Gas6 concentration in PE is unaffected. These data confirm the hypothesis that Gas6 protein is involved in inflammation and in particular in activation of innate immunity. Gas6 testing considerably improved the accuracy of the D-dimer test in PE diagnosis, limiting false positives. However, the clinical utility of plasma Gas6 detection in the differential diagnosis of acute dyspnea in PE evaluation must be confirmed in a larger study.

Table 4 Patient distribution after Gas6 test.

All patients			Diagnosis	Patients with LICP		
$n = 60$				$n = 48$		
Gas6 <27	n	Gas6 ≥27		Gas6 <24	n	Gas6 ≥24
29	29	0	PE	20	20	0
1	11	10	I	0	9	9
6	12	6	HF	5	11	6
8	8	0	N	7	8	1
44 (73%)	60	16 (27%)		32 (67%)	48	16 (33%)

Conflict of interest statement

All authors declare that they have no competing interests.

Ethics statement

All patients were aged >18 years and gave written informed consent. At the beginning of the enrolment a review board was not required for observational studies.

Acknowledgments

The study was supported by Regione Piemonte ''Ricerca sanitaria finalizzata'' no. 1051/2006 and by Università del Piemonte Orientale ''A. Avogadro'' ''Ricerca locale 2005'' research grants.

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